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(21) International Application Number: PCT/GB96/02357 (22) International Filing Date: 23 September 1996 (23.09.96) (30) Priority Data: 9519299.3 21 September 1995 (21.09.95) GB (71) Applicant (for all designated States except US): PROVOST, FELLOWS AND SCHOLARS OF THE COLLEGE OF THE HOLY AND UNDIVIDED TRINITY OF QUEEN ELIZABETH NEAR DUBLIN [IE/IE]; Trinity College Dublin, Dublin 2 (IE). (72) Inventors; and (75) Inventors/Applicants (for US only): FARRAR, Gwentyth, Jane [IE/IE]; 9 The Crescent, Monkstown D20, County Dublin (IE). HUMPHRIES, Peter [GB/IE]; 5 Holmwood, Cabinteely D15, County Dublin (IE). KENNA, Paul, Francis [IE/IE]; 176 New Cabra Road, Dublin 7 (IE). (74) Agent: MURGITROYD & COMPANY; 373 Scotland Street, Glasgow G5 8QA (GB).	(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>	
(54) Title: STRATEGY FOR SUPPRESSING THE EXPRESSION OF AN ENDOGENEOUS GENE BY USING COMPOUNDS THAT ARE ABLE TO BIND TO THE NON-CODING REGIONS OF THE GENE TO BE SUPPRESSED (57) Abstract The invention provides strategy for suppressing expression of an endogenous gene, wherein said strategy comprises providing suppression effectors able to bind to the non-coding regions of a gene to be suppressed, to prevent the functional expression thereof. The suppression effectors may be antisense nucleic acids, and the non-coding regions can include the transcribed but non-translated regions of a gene. The strategy can also introduce a replacement gene.		

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STRATEGY FOR SUPPRESSING THE EXPRESSION OF AN ENDOGENEOUS GENE BY USING
COMPOUNDS THAT ARE ABLE TO BIND TO THE NON-CODING REGIONS OF THE GENE TO
BE SUPPRESSED

✓ Field of the Invention

The present invention relates to a strategy and medicaments for suppressing a gene. In particular the invention relates to the suppression of mutated genes which give rise to a dominant or deleterious effect either monogenically or polygenically. The invention relates to a strategy for suppressing a gene or disease allele such that (if required) a replacement gene, gene product or alternative gene therapy can be introduced.

The invention also relates to a medicament or medicaments for use in suppressing a gene or disease allele which is present in a genome of one or more individuals or animals. The said medicament(s) may also introduce the replacement gene sequence, product or alternative therapy.

Generally the strategy of the present invention will be useful where the gene, which is naturally present in the genome of a patient, contributes to a disease state. Generally, the gene in question will be mutated, that is, will possess alterations in its nucleotide sequence that affect the function or level of the gene product. For example, the alteration may result in an

1 altered protein product from the wild type gene or
2 altered control of transcription and processing.
3 Inheritance or the somatic acquisition of such a
4 mutation can give rise to a disease phenotype or can
5 predispose an individual to a disease phenotype.
6 However the gene of interest could also be of wild type
7 phenotype, but contribute to a disease state in another
8 way such that the suppression of the gene would
9 alleviate or improve the disease state.

10 Background of the Invention

11 Studies of degenerative hereditary ocular conditions,
12 including Retinitis Pigmentosa (RP) and various macular
13 dystrophies have resulted in a substantial elucidation
14 of the molecular basis of these debilitating human eye
15 disorders. In a collaborative study, applying the
16 approach of genetic linkage, two x-linked RP genes were
17 localised to the short arm of the X chromosome (Ott et
18 al. 1990). In autosomal dominant forms of RP (adRP)
19 three genes have been localised. The first adRP gene
20 mapped on 3q close to the gene encoding the
21 photoreceptor specific protein rhodopsin (McWilliam et
22 al. 1989; Dryja et al. 1990). Similarly, an adRP gene
23 was placed on 6p close to the gene encoding the
24 photoreceptor specific protein peripherin/RDS (Farrar
25 et al. 1991a,b; Kajiwara et al. 1991). A third adRP
26 gene mapped to 7q (Jordan et al. 1993); no known
27 candidate genes for RP reside in this region of 7q. In
28 addition, the disease gene segregating in a Best's
29 macular dystrophy family was placed on 11q close to the
30 region previously shown to be involved in some forms of
31 this dystrophy (Mansergh et al. 1995). Recently, an
32 autosomal recessive RP gene was placed on 1q (Van Soest
33 et al. 1994). Genetic linkage, in combination with
34 techniques for rapid mutational screening of candidate
35 genes, enabled subsequent identification of causative
36 mutations in the genes encoding rhodopsin and

1 peripherin/RDS proteins. Globally about 100 rhodopsin
2 mutations have now been found in patients with RP or
3 congenital stationary night blindness. Similarly about
4 40 mutations have been characterised in the
5 peripherin/RDS gene in patients with RP or with various
6 macular dystrophies.

7
8 Knowledge of the molecular aetiology of some forms of
9 human inherited retinopathies has stimulated the
10 establishment of methodologies to generate animal
11 models for these diseases and to explore methods of
12 therapeutic intervention; the goal being the
13 development of treatments for human retinal diseases
14 (Farrar et al. 1995). Surgical procedures enabling the
15 injection of sub-microlitre volumes of fluid
16 intravitally or subretinally into mouse eyes have
17 been developed by Dr^v Paul Kenna. In conjunction with
18 the generation of animal models, optimal systems for
19 delivery of gene therapies to retinal tissues using
20 viral (inter alia Adenovirus, Adeno Associated Virus,
21 Herpes Simplex Type 1 Virus) and non-viral (inter alia
22 liposomes, dendrimers) vectors alone or in association
23 with derivatives to aid gene transfer are being
24 investigated.

25
26 Generally, gene therapies utilising both viral and
27 non-viral delivery systems have been applied in the
28 treatment of a number of inherited disorders; of
29 cancers and of some infectious disorders. The majority
30 of this work has been undertaken on animal models,
31 although, some human gene therapies have been approved.
32 Many studies have focused on recessively inherited
33 disorders, the rationale being, that the introduction
34 and efficient expression of the wild type gene may be
35 sufficient to result in a prevention/amelioration of
36 disease phenotype. In contrast gene therapy for

1 dominant disorders will require the suppression of the
2 dominant disease allele. Notably the majority of
3 characterised mutations that cause inherited retinal
4 degenerations such as RP are inherited in an autosomal
5 dominant fashion. Indeed there are over 1,000 autosomal
6 dominantly inherited disorders in man. In addition
7 there are many polygenic disorders due to the
8 co-inheritance of a number of genetic components which
9 together give rise to a disease phenotype. Effective
10 gene therapy in dominant or polygenic disease will
11 require suppression of the disease allele while in many
12 cases still maintaining the function of the normal
13 allele.

14
15 Strategies to differentiate between normal and disease
16 alleles and to selectively switch off the disease
17 allele using suppression effectors inter alia antisense
18 DNA/RNA, ribozymes or triple helix DNA, targeted
19 towards the disease mutation may be difficult in many
20 cases and impossible in others - frequently the disease
21 and normal alleles may differ by only a single
22 nucleotide. For example, the disease mutation may not
23 occur at a ribozyme cleavage site. Similarly the
24 disease allele may be difficult to target specifically
25 by antisense DNA/RNA or triple helix DNA if there are
26 only small sequence differences between the disease and
27 normal alleles. A further difficulty inhibiting the
28 development of gene therapies is the heterogeneous
29 nature of some dominant disorders - many different
30 mutations in the same gene give rise to a similar
31 disease phenotype. The development of specific gene
32 therapies for each of these would be extremely costly.
33 To circumvent the dual difficulties associated with
34 specifically targeting the disease mutation and the
35 genetic heterogeneity present in some inherited
36 disorders, the present invention aims to provide a

1 novel strategy for gene suppression and replacement
2 exploiting the noncoding and control regions of a gene.
3
4 Suppression effectors have been used previously to
5 achieve specific suppression of gene expression.
6 Antisense DNA and RNA has been used to inhibit gene
7 expression in many instances. Many modifications, such
8 as phosphorothioates, have been made to antisense
9 oligonucleotides to increase resistance to nuclease
10 degradation, binding affinity and uptake (Cazenave et
11 al. 1989; Sun et al. 1989; McKay et al. 1996; Wei et
12 al. 1996). In some instances, using antisense and
13 ribozyme suppression strategies has ^{led} led to the reversal
14 of the tumour phenotype by greatly reducing the
15 expression of a gene product or by cleaving a mutant
16 transcript at the site of the mutation (Carter and
17 Lemoine 1993; Lange et al. 1993; Valera et al. 1994;
18 Dosaka-Akita et al. 1995; Feng et al. 1995; Quattrone
19 et al. 1995; Ohta et al. 1996). For example, neoplastic
20 reversion was obtained using a ribozyme targeted to the
21 codon 12 H-ras mutation in bladder carcinoma cells
22 (Feng et al. 1995). Ribozymes have also been proposed
23 as a means of both inhibiting gene expression of a
24 mutant gene and of correcting the mutant by targeted
25 trans-splicing (Sullenger and Cech 1994; Jones et al.
26 1996). Ribozymes can be designed to elicit
27 autocatalytic cleavage of RNA targets. However the
28 inhibitory effect of some ribozymes may be due in part
29 to an antisense effect of the variable antisense
30 sequences flanking the catalytic core which specify the
31 target site (Ellis and Rodgers 1993; Jankowsky and
32 Schwenzer 1996). Ribozyme activity may be augmented by
33 the use of non-specific nucleic acid binding proteins
34 or facilitator oligonucleotides (Herschlag et al. 1994;
35 Jankowsky and Schwenzer 1996). Triple helix approaches
36 have also been investigated for sequence specific gene

1 suppression - triplex forming oligonucleotides have
2 been found in some cases to bind in a sequence specific
3 manner (Postel et al. 1991; Duval-Valentin et al. 1992;
4 Hardenbol and Van Dyke 1996; Porumb et al. 1996).
5 Similarly peptide nucleic acids have been shown in some
6 instances to inhibit gene expression (Hanvey et al.
7 1992; Knudson and Nielsen 1996). Minor groove binding
8 polyamides have been shown to bind in a sequence
9 specific manner to DNA targets and hence may represent
10 useful small molecules for future suppression at the
11 DNA level (Trauger et al. 1996). In addition,
12 suppression has been obtained by interference at the
13 protein level using dominant negative mutant peptides
14 and antibodies (Herskowitz 1987; Rimsky et al. 1989;
15 Wright et al. 1989). In some cases suppression
16 strategies have lead to a reduction in RNA levels
17 without a concomitant reduction in proteins, whereas in
18 others, reductions in RNA levels have been mirrored by
19 reductions in protein levels.

20

21 The present invention aims to circumvent the
22 shortcomings in the prior art by using a two step
23 approach for suppression and replacement.

24

25 According to the present invention there is provided a
26 strategy for suppressing expression of an endogenous
27 gene, wherein said strategy comprises providing
28 suppression effectors able to bind to the non-coding
29 regions of a gene to be suppressed, to prevent the
30 functional expression thereof. Preferably the
31 suppression effectors are antisense nucleic acids.
32 Preferably the targetted non-coding regions include the
33 transcribed but non-translated regions of a gene.

34

35 Generally the term suppression effectors includes
36 nucleic acids, peptide nucleic acids (PNAs) or peptides

1 which can be used to silence or reduce gene expression
2 in a sequence specific manner.

3
4 The antisense nucleic acids can be DNA or RNA, can be
5 directed to 5' and/or 3' untranslated regions and/or to
6 introns and/or to control regions or to any combination
7 of such untranslated regions. However targetted the
8 binding of the antisense nucleic acid prevents or
9 lowers the functional expression of the endogenous
10 gene. Chimeric antisense nucleic acids including a
11 small proportion of translated regions of a gene can be
12 used in some cases to help to optimise suppression.
13 Likewise Chimeric antisense nucleic acids including a *lower cost*
14 small proportion of promoter regions of a gene can be
15 used in some cases to help to optimise suppression.

16
17 Generally the term 'functional expression' means the
18 expression of a gene product able to function in a
19 manner equivalent to or better than a wild type
20 product. In the case of a mutant gene 'functional
21 expression' means the expression of a gene product
22 whose presence gives rise to a deleterious effect.

23
24 In a particular embodiment of the invention the
25 strategy further employs ribozymes. These can be
26 designed to elicit cleavage of target RNAs.

27
28 The strategy further employs nucleotides which form
29 triple helix DNA.

30
31 Nucleic acids ~~for~~ for antisense, ribozymes and triple
32 helix may be modified to increase stability, binding
33 efficiencies and uptake as discussed earlier. Nucleic
34 acids can be incorporated into a vector. Vectors
35 include DNA plasmid vectors, RNA or DNA virus vectors.
36 Th se can be combined with lipids, polymers or other

1 derivatives to aid gene delivery and expression.

2

3 The invention further provides the use of antisense
4 nucleotides, ribozymes, triple helix nucleotides or
5 other suppression effectors alone or in a vector or
6 vectors, wherein the nucleic acids are able to bind
7 specifically to untranslated regions of a gene such as
8 the 5' and 3' UTRs to prevent the functional expression
9 thereof, in the preparation of a medicament for the
10 treatment of an autosomal dominant disease.

11

12 In a further embodiment the non-coding regions of the
13 gene can include promoter regions which are
14 untranslated.

15

16 According to the present invention there is provided a
17 strategy for suppressing an endogenous gene and
18 introducing a replacement gene, said strategy
19 comprising the steps of:

20

21 1. providing antisense nucleic acid able to bind to at
22 least one non-coding or untranslated region of a gene
23 to be suppressed and

24

25 2. providing genomic DNA or cDNA encoding a replacement
26 gene sequence,

27

28 wherein the antisense nucleic acid is unable to bind to
29 equivalent non-coding or untranslated regions in the
30 genomic DNA or cDNA to prevent expression of the
31 replacement gene sequence.

32

33 The replacement nucleic acids will not be recognised by
34 the suppression nucleic acid. The control sequences of
35 the replacement nucleic acid may belong to a different
36 mammalian species, may belong to a different human gene

1 or may be similar but altered from those in the gene to
2 be suppressed and may thus permit translation of the
3 part of the replacement nucleic acid to be initiated.

4
5 By control sequences is meant sequences which are
6 involved in the control of gene expression or in the
7 control of processing and/or sequences present in
8 mature RNA transcripts and/or in precursor RNA
9 transcripts, but not including protein coding
10 sequences.

11
12 In a particular embodiment of the invention there is
13 provided a strategy for gene suppression targeted
14 towards the non-coding regions of a gene and using a
15 characteristic of one of the alleles of a gene, for
16 example, the allele carrying a disease mutation.
17 Suppressors are targeted to non-coding regions of a
18 gene and to a characteristic of one allele of a gene
19 such that suppression is specific or partially specific
20 to one allele of the gene. The invention further
21 provides for replacement nucleic acids containing
22 altered non-coding sequences such that replacement
23 nucleic acids cannot be recognised by suppressors which
24 are targeted towards the non-coding regions of a gene.
25 Replacement nucleic acids provide the wild type or an
26 equivalent gene product but are protected completely or
27 in part from suppression effectors targeted to non-
28 coding regions.

29
30 In a further embodiment of the invention there is
31 provided replacement nucleic acids with altered non-
32 coding sequences such that replacement nucleic acids
33 cannot be recognised by naturally occurring endogenous
34 suppressors present in one or more individuals, animals
35 or plants. Replacement nucleic acids with altered non-
36 coding sequences provide the wild type or equivalent

1 gene product but are completely or partially protected
2 from suppression by naturally occurring endogenous
3 suppression effectors.

4
5 In an additional embodiment of the invention there is
6 provided replacement nucleic acids with altered non-
7 coding sequences such that replacement nucleic acids
8 provide a wild type or equivalent gene product or gene
9 product with beneficial characteristics. For example,
10 the 3' non-coding sequences of the replacement nucleic
11 acids could be altered to modify the stability and turn
12 over the RNA expressed from the replacement nucleic
13 acids thereby sometimes affecting levels of ~~resulting~~ resulting
14 gene product.

15
16 The invention further provides the use of a vector or
17 vectors containing suppression effectors in the form of
18 nucleic acids, said nucleic acids being directed
19 towards untranslated regions or control sequences of
20 the target gene and vector(s) containing genomic DNA or
21 cDNA encoding a replacement gene sequence to which
22 nucleic acids for suppression are unable to bind, in
23 the preparation of a combined medicament for the
24 treatment of an autosomal dominant disease. Nucleic
25 acids for suppression or replacement gene nucleic acids
26 may be provided in the same vector or in separate
27 vectors. Nucleic acids for suppression or replacement
28 gene nucleic acids may be provided as a combination of
29 nucleic acids alone or in vectors. The vector may
30 contain antisense nucleic acid with or without,
31 ribozymes.

32
33 The invention further provides a method of treatment
34 for a disease caused by an endogenous mutant gene, said
35 method comprising sequential or concomitant
36 introduction of (a) antisense nucleic acids to the

1 non-coding regions of a gene to be suppressed; to the
2 5' and/or 3' untranslated regions of a gene or intronic
3 regions or to the non-control regions of a gene to be
4 suppressed, (b) replacement gene sequence with control
5 sequences which allow it to be expressed.

6
7 The nucleic acid for gene suppression can be
8 administered before or after or at the same time as the
9 replacement gene is administered.

10
11 The invention further provides a kit for use in the
12 treatment of a disease caused by an endogenous mutation
13 in a gene, the kit comprising nucleic acids for
14 suppression able to bind to the 5' and / or 3'
15 untranslated regions or intronic regions or control
16 regions of the gene to be suppressed and (preferably
17 packaged separately thereto) a replacement nucleic acid
18 to replace the mutant gene having a control sequence to
19 allow it to be expressed.

20
21 Nucleotides can be administered as naked DNA or RNA,
22 with or without ribozymes and/or with dendrimers.
23 Ribozymes stabilise DNA and block transcription.
24 Dendrimers (for example dendrimers of
25 methylmethacrylate) can be utilised, it is believed the
26 ~~dendrimers~~ ^{v dendrimers} mimic histones and as such are capable of
27 transporting nucleic acids into cells.
28 Oligonucleotides can be synthesized, purified and
29 modified with phosphorothioate linkages and 2'-O-allyl
30 groups to render them resistant to cellular nucleases
31 while still supporting RNase H mediated degradation of
32 RNA. Also, ~~nucleic~~ ^{v nucleic} acids can be mixed with lipids to
33 increase efficiency of delivery to somatic tissues.

34
35 Nucleotides can be delivered in vectors. Naked nucleic
36 acids or nucleic acids in vectors can be delivered with

1 lipids or other derivatives which aid gene delivery.
2 Nucleotides may be modified to render them more stable,
3 for example, resistant to cellular nucleases while
4 still supporting RNaseH mediated degradation of RNA or
5 with increased binding efficiencies as discussed
6 earlier.

7
8 Suppression effectors and replacement sequences can be
9 injected sub-sectionally, or may be administered
10 systemically.

11
12 There is now an armament with which to obtain gene
13 suppression. This, in conjunction with a better
14 understanding of the molecular etiology of disease,
15 results in an ever increasing number of disease targets
16 for therapies based on suppression. In many cases,
17 complete (100%) suppression of gene expression has been
18 difficult to achieve. Possibly a combined approach
19 using a number of suppression effectors may be
20 required. For some disorders it may be necessary to
21 block expression of a disease allele completely to
22 prevent disease symptoms whereas for others low levels
23 of mutant protein may be tolerated. In parallel with an
24 increased knowledge of the molecular defects causing
25 disease has been the realisation that many disorders
26 are genetically heterogeneous. Examples in which
27 multiple genes and/or multiple mutations within a gene
28 can give rise to a similar disease phenotype include
29 osteogenesis imperfecta, familial hypercholesterolemia,
30 retinitis pigmentosa, and many others.

31
32 The invention addresses some shortcomings of the prior
33 art and aims to provide a novel approach to the design
34 of suppression effectors directed to target mutant
35 genes. Suppression of every mutation giving rise to a
36 disease phenotype may be costly, problematic and

1 sometimes impossible. Disease mutations are often
2 single nucleotide changes. As a result differentiating
3 between the disease and normal alleles may be
4 difficult. Furthermore some suppression effectors
5 require specific sequence targets, for example,
6 ribozymes can only cleave at NUX sites and hence will
7 not be able to target some mutations. Notably, the wide
8 spectrum of mutations observed in many diseases adds an
9 additional layer of complexity in the development of
10 therapeutic strategies for such disorders. A further
11 problem associated with suppression is the high level
12 of homology present in coding sequences between members
13 of some gene families. This can limit the range of
14 target sites for suppression which will enable specific
15 suppression of a single member of such a gene family.

16
17 The strategy described herein has applications for
18 alleviating autosomal dominant diseases. Complete
19 silencing of a disease allele may be difficult to
20 achieve using antisense, ribozyme and triple helix
21 approaches or any combination of these. However small
22 quantities of mutant product may be tolerated in some
23 autosomal dominant disorders. In others a significant
24 reduction in the proportion of mutant to normal product
25 may result in an amelioration of disease symptoms.
26 Hence this strategy may be applied to any autosomal
27 dominantly inherited disease in man where the molecular
28 basis of the disease has been established. This
29 strategy will enable the same therapy to be used to
30 treat a wide range of different disease mutations
31 within the same gene. The development of strategies
32 will be important to future gene therapies for some
33 autosomal dominant diseases, the key to a general
34 strategy being that it circumvents the need for a
35 specific therapy for every dominant mutation in a given
36 disease-causing gene. This is particularly relevant in

1 some disorders, for example, rhodopsin linked autosomal
2 dominant RP (adRP), in which to date about 100
3 different mutations in the rhodopsin gene have been
4 observed in adRP patients. The costs of developing
5 designer therapies for each individual mutation which
6 may be present in some cases in a single patient are
7 prohibitive at present. Hence strategies such as this
8 using a more universally applicable approach for
9 therapy will be required.

10

11 This strategy may be applied in gene therapy approaches
12 for biologically important polygenic disorders
13 affecting large proportions of the world's populations
14 such as age related macular degeneration (ARMD),
15 glaucoma, manic depression, cancers having a familial
16 component and indeed many others. Polygenic diseases
17 require the inheritance of more than one mutation
18 (component) to give rise to the disease phenotype.
19 Notably an amelioration in disease symptoms may require
20 reduction in the presence of only one of these
21 components, that is, suppression of one of the
22 genotypes which, together with others, leads to the
23 disease phenotype, may be sufficient to prevent or
24 ameliorate symptoms of the disease. In some cases the
25 suppression of more than one component giving rise to
26 the disease pathology may be required to obtain an
27 amelioration in disease symptoms. The strategy
28 described here may be applied broadly to possible
29 future interventive therapies in common polygenic
30 diseases to suppress a particular genotype(s) and
31 thereby suppress the disease phenotype.

32

33 In the present invention suppression effectors are
34 designed specifically to target the non-coding regions
35 of genes, for example, the 5' and 3' UTRs. This
36 provides sequence specificity for gene suppression. In

1 addition it provides greater flexibility in the choice
2 of target sequence for suppression in contrast to
3 suppression strategies directed towards single disease
4 mutations. Furthermore it allows suppression effectors
5 to target non-coding sequences 5' or 3' of the coding
6 region thereby allowing the possibility of including
7 the ATG start site in the target site for suppression
8 and hence presenting an opportunity for suppression at
9 the level of translation or inducing instability in RNA
10 by, for example, cleavage of the RNA before the polyA
11 tail. Notably the invention has the advantage that the
12 same suppression strategy when directed to the 5' and
13 3' non-coding sequences could be used to suppress, in
14 principle, any mutation in a given gene. This is
15 particularly relevant when large numbers of mutations
16 within a single gene cause a disease pathology.
17 Suppression targeted to non-coding sequences allows,
18 when necessary, the introduction of a replacement
19 gene(s) with the same or similar coding sequences to
20 provide the normal gene product. The replacement gene
21 can be designed to have altered non-coding sequences
22 and hence can escape suppression as it does not contain
23 the target site(s) for suppression. The same
24 replacement gene could in principle be used in
25 conjunction with the suppression of any disease
26 mutation in a given gene. In the case of suppression of
27 an individual member of a gene family, the non-coding
28 regions typically show lower levels of homology between
29 family members thereby providing more flexibility and
30 specificity in the choice of target sites for
31 suppression. In relation to this aspect of the
32 invention, the use of intronic sequences for
33 suppression of an individual member of a family of
34 genes has been described in a previous invention (REF:
35 WO 92/07071). However the use of 5' and 3' non-coding
36 sequences as targets for suppression holds the

1 advantage that these sequences are present not only in
2 precursor messenger RNAs but also in mature messenger
3 RNAs, thereby enabling suppressors to target all forms
4 of RNA. In contrast, intronic sequences are spliced out
5 of mature RNAs.

6
7 In summary the invention can involve gene suppression
8 and replacement such that the replacement gene cannot
9 be suppressed. Both the same suppression and
10 replacement steps can be used for many and in some
11 cases all of the disease mutations identified in a
12 given gene. Therefore the invention enables the same
13 approach to be used to suppress a wide range of
14 mutations within the same gene. Suppression and
15 replacement can be undertaken in conjunction with each
16 other or separately.

17
18 **Examples**

19
20 The present invention is exemplified using four
21 different genes: human rhodopsin, human peripherin,
22 mouse rhodopsin and mouse peripherin. While all four
23 genes are retinal specific there is no reason why the
24 present invention could not be deployed in the
25 suppression of other genes. Notably the 5'UTR and part
26 of the coding sequence of the COL1A2 gene has been
27 cloned together with a ribozyme to target the 5'UTR of
28 the gene ^{emphasizing} ~~emphasizing~~ the broad utility of the invention
29 in gene suppression. The 5'UTR and part of the coding
30 sequence of the COL1A2 gene in which there are many
31 mutations have previously been identified which give
32 rise to autosomal dominant osteogenesis imperfecta, has
33 begun but was not completed at the time of submission.
34 Many examples of mutant genes which give rise to
35 disease phenotypes are available from the prior art -
36 these all represent disease targets for this invention.

1 The present invention is exemplified using ribozymes
2 with antisense arms to elicit RNA cleavage. There is no
3 reason why other suppression effectors directed towards
4 the non-coding regions of genes could not be used to
5 achieve gene suppression. Many examples from the prior
6 art detailing the use of suppression effectors inter
7 alia antisense RNA/DNA, triple helix, PNAs, peptides to
8 achieve suppression of gene expression are reported as
9 discussed earlier. The present invention is exemplified
10 using ribozymes with antisense arms to elicit cleavage
11 of template RNA transcribed from one vector and
12 non-cleavage of replacement RNAs with altered
13 untranslated region sequences transcribed from a second
14 vector. There is no reason why both the suppression and
15 replacement steps could not be in the same vector. In
16 addition there is no reason why ribozymes could not be
17 used to combine both the suppression and replacement
18 steps, that is, to cleave the target RNA and to ligate
19 to the cleavage product, a replacement RNA with an
20 altered sequence, to prevent subsequent cleavage by
21 ribozymes which are frequently autocatalytic as
22 discussed. The present invention is exemplified using
23 suppression effectors directed to target the 5'
24 untranslated region of the above named genes. There is
25 no reason why other non-coding regions of a gene inter
26 alia the 3' untranslated region or the regions involved
27 in the control of gene expression such as promoter
28 regions or any combination of non-coding regions could
29 not be used to achieve gene suppression. Suppression
30 targeted to any non-coding region of a gene would allow
31 the expression of a replacement gene with altered
32 sequences in the non-coding region of the gene to which
33 the suppression effector(s) was targeted.

34

35 **MATERIALS AND METHODS**

36

1 **Cloning vectors**

2
3 cDNA templates, cDNA hybrids with altered non-coding
4 sequences, ribozymes and antisense DNA fragments were
5 cloned into commercial expression vectors (~~pCDNA3~~ ^{pCDNA3},
6 pZeoSV or pBluescript) which enable expression in a
7 test tube from T7, T3 or SP6 promoters or expression in
8 cells from CMV or SV40 promoters. Inserts were placed
9 into the multiple cloning site (MCS) of these vectors
10 typically at or near the terminal ends of the MCS to
11 delete most of the MCS and thereby prevent any possible
12 problems with efficiency of expression subsequent to
13 cloning.

14
15 **Sequencing protocols**

16
17 Clones containing template cDNAs, hybrid cDNAs with
18 altered non-coding sequences, ribozymes and antisense
19 were sequenced by ABI automated sequencing machinery
20 using standard protocols.

21
22 **Expression of RNAs**

23
24 RNA was obtained from clones in vitro using a
25 commercially available Ribomax expression system
26 (Promega) and standard protocols. RNA purifications
27 were undertaken using the Bio-101 RNA purification^{purification} kit
28 or a solution of 0.3M sodium acetate and 0.2% SDS.
29 Cleavage reactions were performed using standard
30 protocols with varying MgCl₂ concentrations (0-15mM) at
31 37°C typically for 3 hours. Time points were performed
32 at the predetermined optimal MgCl₂ concentrations for up
33 to 5 hours. Radioactively labeled RNA products were
34 obtained by incorporating ^{α-³²P} rUTP (Amersham) in the
35 expression reactions (Gaughan et al. 1995). Labeled RNA
36 products were run on polyacrylamide gels before

1 cleavage reactions were undertaken for the purposes of
2 RNA purification and subsequent to cleavage reactions
3 to establish if RNA cleavage had been achieved.

4
5 The exact base at which transcription starts has not
6 been defined fully for some promoters (pcDNA3
7 Invitrogen) hence the sizes of the RNA products may
8 vary slightly from those predicted in Table 1. In
9 addition ^{multiple} ~~multiple~~ rounds of cloning of a cDNA results is
10 inserts carrying extra portions of MCS again, sometimes
11 altering marginally the size of expressed RNA products.
12 Typically 4-8% polyacrylamide gels were run to resolve
13 RNA products.

14 15 RNA secondary structures

16
17 Predictions of the secondary structures of human
18 rhodopsin, mouse rhodopsin, human peripherin, mouse
19 peripherin and human type I Collagen COLIA2 mRNAs where
20 obtained using the RNAPlotFold program. Ribozyme and
21 antisense was designed to target areas of the RNA that
22 were predicted to be accessible to suppression
23 effectors and which were composed of non-coding
24 sequence. The integrity of open loop structures was
25 evaluated from the 15 most probable RNA structures.
26 Additionally RNA structures for truncated RNA products
27 were generated and the ^{integrity} ~~integrity~~ of open loops between
28 full length and truncated RNAs compared.

29 30 TEMPLATE/HYBRID/RIBOZYME AND ANTISENSE CONSTRUCTS

31 32 Examples

33
34 Various products of the examples are illustrated in
35 Figures 1 to 20 and are explained in the results
36 sections.

1 **Sequences**

2

3 In each case the most relevant sequences have been
4 underlined. The position of the ATG start in each
5 sequence is highlighted by an arrow. Sequences 1 to 18
6 below are represented in Figures 21 to 39 respectively.

7

8 Sequence 1: Mouse ~~Rhodopsin~~ ^{Rhodopsin} cDNA sequences
9 ~~mouse~~ ^{mouse} rhodopsin 5'UTR sequences/the ATG
10 start site/mouse rhodopsin coding
11 sequences are shown.

12

13 Sequence 2: Mouse Rhodopsin cDNA with altered non
14 (F+R) -coding sequences
15 mouse rhodopsin 5'UTR sequences with a
16 1 base change/the ATG start site/mouse
17 rhodopsin coding sequences are shown.

18

19 Sequence 3: Mouse Rhodopsin cDNA with altered non-
20 (F+R) coding sequences
21 mouse rhodopsin 5'UTR sequences with a
22 1 base change/the ATG start site/mouse
23 rhodopsin coding sequences are shown.

24

25 Sequence 4: Ribozyme 3

26

27 Sequence 5: Human Rhodopsin cDNA sequence
28 human rhodopsin 5'UTR sequences/the ATG
29 start site/human rhodopsin coding
30 sequences are shown.

31

32 Sequence 6: Human Rhodopsin cDNA with altered non-
33 coding sequences
34 human rhodopsin 5'UTR sequences
35 (shorter UTR)/the ATG start site/human
36 rhodopsin coding sequences are shown.

(SEQ ID NO: 1-18,
respectively)

- 1 Sequence 7: Ribozyme 15
- 2
- 3 Sequence 8: Mouse ~~peripherin~~^{peripherin} cDNA sequences
- 4 mouse peripherin 5'UTR sequences/the
- 5 ATG start site/mouse peripherin coding
- 6 sequences are shown.
- 7
- 8 Sequence 9: Mouse ~~peripherin~~^{peripherin} cDNA with altered non-
- 9 coding sequences
- 10 mouse rhodopsin 5'UTR sequences/the ATG
- 11 start site/mouse peripherin coding
- 12 sequences are shown.
- 13
- 14 Sequence 10: Ribozyme 17
- 15
- 16 Sequence 11: Human peripherin cDNA sequences
- 17 human peripherin 5'UTR sequences/the
- 18 ATG start site/human peripherin coding
- 19 sequences are shown.
- 20
- 21 Sequence 12: Human peripherin cDNA with altered non-
- 22 coding sequences
- 23 Partial human and mouse peripherin
- 24 5'UTR sequences/the ATG start
- 25 site/human peripherin coding sequences
- 26 are shown.
- 27
- 28 Sequence 13: Ribozyme 8
- 29
- 30 Sequence 14: Ribozyme 9
- 31
- 32 Sequence 15: Human type I collagen (~~corin~~^{cd-1A2}) sequence
- 33 - 5'UTR and exon 1 sequence
- 34
- 35 Sequence 16: Ribozyme 18
- 36

1 Sequence 17: Antisense construct containing 127bp of
2 antisense ~~sequence~~^{sequence} targeting the 5'UTR
3 of the mouse peripherin gene.
4

5 Sequence 18: Sense construct containing 127bp of
6 sense sequence from the 5'UTR of the
7 mouse peripherin gene.
8

9 **Mouse Rhodopsin**

10

11 **Template cDNA**

12 A full length mouse rhodopsin cDNA was generated from a
13 partial cDNA clone missing the sequence coding for the
14 first 20 amino acids of the protein and a partial
15 genomic clone which enabled the production of a full
16 length cDNA (kindly donated by Dr. Wolfgang Baehr). The
17 full length cDNA was cloned into the EcoRI site of
18 [pcDNA3] in a 5' to 3' orientation allowing subsequent
19 expression of RNA from the T7 or CMV promoters in the
20 vector. The full length 5'UTR sequence was present in
21 this clone. In addition to the full length 5' UTR
22 sequence the clone contains additional 5' upstream
23 sequence of the mouse rhodopsin gene as the clone was
24 generated using the EcoRI site present at position 1120
25 (Accession number: M55171). (Sequence 1)
26

27 **Hybrid cDNAs with altered non-coding regions**

28

29 **Hybrid I**

30 The mouse rhodopsin hybrid cDNA sequence was altered in
31 the non-coding sequences by PCR primer directed
32 mutagenesis and cloned into the HindIII and EcoRI sites
33 of [pcDNA3] in a 5' to 3' orientation allowing subsequent
34 expression of RNA from the T7 or CMV promoters in the
35 vector. PCR mutagenesis was undertaken using a HindIII
36 (in the MCS of pcDNA3) to Eco47111 (in Exon 2 of the

1 gene) DNA fragment. The 5'UTR was altered significantly
 2 - the mouse rhodopsin 5'UTR was completely replaced by
 3 the 5'UTR of the human peripherin gene, that is, by
 4 5'UTR sequence from a different gene (peripherin) and
 5 from a different species (human) but from a gene
 6 expressed in the same tissue as mouse rhodopsin, i.e.,
 7 photoreceptor cells (Sequence 2). The sequence of the
 8 mouse rhodopsin cDNA is present in the clone from the
 9 ATG start onwards.

10

11 Hybrid 2

12 The mouse rhodopsin hybrid cDNA sequence was altered in
 13 the non-coding sequences to eliminate the GUC ribozyme
 14 binding site targeted in the 5'UTR of mouse rhodopsin.
 15 The U of the target was changed to G, that is, No. 1
 16 GUC-->GGC (Sequence 3). Again PCR mutagenesis was
 17 primer driven and was undertaken using a HindIII (in
 18 pCDNA3) to Eco47111 (in the coding sequence of the
 19 mouse rhodopsin cDNA) DNA fragment.

20

21 Ribozyme constructs

22 A hammerhead ribozyme (termed Rib3) designed to target
 23 an open loop structure in the RNA in the 5' non-coding
 24 region of the gene was cloned into the HindIII and XbaI
 25 sites of pCDNA3 again allowing subsequent expression of
 26 RNA from the T7 or CMV promoters in the vector.
 27 (Sequence 4). The target site was GUC at position
 28 1393-1395 of the mouse rhodopsin sequence (Accession
 29 number: M55171). Antisense flanks are underlined.
 30 Rib3: CUUCGUACUGAUGAGUCCGUGAGGACGAAACAGAGAC (nucleotides 95-131
 31 of SEQ ID NO: 4)

32

32 Human Rhodopsin

33

34 Template cDNA

35 The human rhodopsin cDNA was cloned into the HindIII
 36 and EcoRI sites of the MCS of pCDNA3 in a 5' to 3'

1 orientation allowing subsequent expression of RNA from
 2 the T7 or CMV promoters in the vector. The full length
 3 5'UTR sequence was inserted into this clone using ^{PCDNA3}
 4 primer driven PCR mutagenesis and a ^{HindIII} ~~HindIII~~ (in ^{PCDNA3} ~~PCDNA3~~)
 5 to BstEII (in the coding sequence of the human
 6 rhodopsin cDNA) DNA fragment (^{SEQ ID NO: 5} Sequence 5)

7
 8 Hybrid cDNAs with altered non-coding regions ^{altered}
 9 The human rhodopsin hybrid cDNA with ^{altered} ~~altered~~ non-coding
 10 sequences was cloned into the EcoRI site of ^{PCDNA3} ~~PCDNA3~~ in a
 11 5' to 3' orientation allowing subsequent expression of
 12 RNA from the T7 or CMV promoters in the vector. The
 13 5'UTR of this clone included only the first 21 bases of
 14 the non-coding region of human rhodopsin before the ATG
 15 start site (^{SEQ ID NO: 6} Sequence 6).

16 Ribozye constructs

17 A hammerhead ribozyme (termed Rib15) designed to target
 18 an open loop structure in the RNA from the non-coding
 19 regions of the gene was cloned subsequent to synthesis
 20 and annealing into the ^{HindIII} ~~HindIII~~ and ^{XbaI} ~~XbaI~~ sites of ^{PCDNA3} ~~PCDNA3~~
 21 again allowing subsequent expression of RNA from the T7
 22 or CMV promoters in the vector (^{SEQ ID NO: 7} Sequence 7). The target
 23 site was AUU (the NUX rule) at position 249-251 of the
 24 human rhodopsin sequence (Accession number: K02281).
 25 Antisense flanks are underlined. ^RRib15:

26 ACCCAAGCUGAUGAGUCCGUGAGGACGAAAUGCUGC (nucleotides 104-139 of ^{SEQ ID NO: 7} Sequence 7)

28 Mouse Peripherin

30 Template cDNA

31 A mouse peripherin cDNA was cloned into the ^{HindIII} ~~HindIII~~ and
 32 EcoRV sites of ^{PCDNA3} ~~PCDNA3~~. The clone is in a 5' to 3'
 33 orientation allowing subsequent expression of RNA from
 34 the T7 or CMV promoters in the vector (^{SEQ ID NO: 8} Sequence 8). The
 35 clone contains the complete 5'UTR sequence together

1 with 27 bases of additional sequence 5' of this UTR
 2 sequence left probably from other cloning vectors.
 3
 4 Hybrid cDNAs with altered non-coding regions
 5 The mouse peripherin hybrid cDNA was altered in the
 6 5'-non-coding region. Using primer driven PCR
 7 mutagenesis the mouse rhodopsin 5'UTR sequence was
 8 replaced by the sequence of the mouse peripherin 5'UTR
 9 (SEQ ID NO: 9). The PCR mutagenesis was achieved using a
 10 HindIII (in pCDNA3) to SacII (in the coding sequence of
 11 the mouse peripherin cDNA) DNA fragment.

12 Ribozyme constructs

14 A hammerhead ribozyme (termed Rib17) designed to target
 15 an open loop structure in the RNA from the non-coding
 16 regions of the gene was cloned into the HindIII and
 17 XbaI sites of pCDNA3 again allowing subsequent
 18 expression of RNA from the T7 or CMV promoters in the
 19 vector (SEQ ID NO: 10). The target site was AUU at
 20 position 162-164 of the mouse peripherin sequence
 21 (Accession number: X14770). Antisense flanks are
 22 underlined. Rib17:
 23 CACUCCUCUGAUGAGUCCGUGAGGACGAAAUCCGAGU (nucleotides 99-136 of
 24 SEQ ID NO: 10)

25 Antisense constructs

26 Antisense and sense constructs were PCR amplified and
 27 cloned into pCDNA3 and pZEOSV for expression in vitro *italics*
 28 and in vivo *italics*. For example, a 127bp fragment from the
 29 5'UTR sequence of mouse peripherin was cloned in both
 30 orientations into the above stated vectors. The
 31 effectiveness of antisense at suppression is under
 32 evaluation. The altered hybrid cDNA clones are being
 33 used to establish if RNAs expressed from these altered
 34 clones are protected from antisense suppression effects
 35 (SEQ ID NOs: 17 and 18).
 36

1 **Human Peripherin**

2

3 **Template cDNA**

4 A human peripherin cDNA cloned into the EcoRI site of
5 the commercially available vector pBluescript was
6 kindly provided by Dr. Gabriel Travis. The clone is in a
7 5' to 3' orientation allowing subsequent expression of
8 RNA from the T7 promoter in the vector. The full length
9 5'UTR sequence is present in this clone (SEQ ID NO: 11).
10

10

11 **Hybrid cDNAs with altered non-coding regions**

12 The hybrid clone with altered non-coding sequences was
13 generated as follows. The hybrid clone contains human
14 RDS 5'UTR sequences until the BamHI site in the human
15 peripherin 5'UTR sequence. From this site the clone
16 runs into mouse RDS 5'UTR sequence until the ATG start
17 site where it returns to human RDS sequence (SEQ ID NO: 12).
18 The clone was generated using primer driven PCR
19 mutagenesis of a BamHI (in the 5'UTR sequence) to BglI
20 (in the coding sequence of the human peripherin cDNA)
21 DNA fragment.
22

22

23 **Ribozyme constructs**

24 Hammerhead ribozymes (termed Rib8 and Rib9) designed to
25 target open loop structures in the RNA from the non-
26 coding regions of the gene were cloned into the HindIII
27 and XbaI sites of pCDNA 3 which again allows subsequent
28 expression of RNA from the T7 or CMV promoters in the
29 vector (Sequences 13 and 14). The target sites were CUA
30 and GUU at positions 234-236 and 190-192 respectively
31 of the human peripherin sequence (Accession number:
32 M62958). Rib8: CCAAGUGCUGAUGAGUCCGUGAGGACGAAAGUCCGG (nucleotides 93-128 of
33 Rib9: CAAACCUUCUGAUGAGUCCGUGAGGACGAAACGAGCC Antisense
34 flanks are underlined. (nucleotides 94-130 of SEQ ID NO: 13)
35

36 **Human Type I Collagen - COL1A2**

1 Template cDNA

2 A partial human type I collagen 1A2 cDNA sequence
 3 including the 5'UTR sequence and exon 1 was cloned ^{Hand III} ^{Xho I}
 4 after PCR amplification into the ^{Hand III} and ^{Xho I} sites
 5 of ^{pcDNA3} ~~pcDNA3~~. The clone is in a 5' to 3' orientation
 6 allowing subsequent expression of RNA from the T7 and
 7 or CMV promoters in the vector ^{SEQ ID NO: 15} (Sequence 15). The clone
 8 contains the complete 5'UTR sequence together with Exon
 9 I of COL1A2.

10
11 Ribozyme ~~construct~~ ^{constructs}

12 A hammerhead ribozyme (termed Rib18) designed to target
 13 an open loop structure in the RNA from the non-coding
 14 regions of the gene was cloned into the ^{Hand III} ^{Xho I}
 15 ^{Xho I} sites of ^{pcDNA3} ~~pcDNA3~~ again allowing subsequent
 16 expression of RNA from the T7 or CMV promoters in the
 17 ^{vector} ^{SEQ ID NO: 16} (Sequence 16). The target site was GUC at
 18 position 448-450 of the human type I collagen 1A2
 19 sequence (Accession number: J03464; M18057; X02488).
 20 Antisense flanks are underlined. ^{TP} Rib18:

21 AGACAUGCCUGAUGAGUCCGUGAGGACGAAACUCCUU (nucleotides 85-121 of SEQ ID NO: 16)

22
23 RESULTS

24
 25 Human and mouse rhodopsin and peripherin cDNAs were
 26 expressed in vitro. ^{halves} Likewise human and mouse rhodopsin
 27 and peripherin cDNAs with altered 5' non-coding
 28 sequences were expressed in vitro. ^{halves} Ribozymes targeting
 29 the 5'UTRs of these retinal cDNAs were also expressed
 30 in vitro. cDNA clones were cut with various restriction
 31 enzymes resulting in the production of differently
 32 sized RNAs after expression. This aided in
 33 differentiating between RNAs expressed from the
 34 original cDNAs or from altered hybrid cDNAs. The sites
 35 used to cut each clone, the predicted sizes of the
 36 resulting RNAs and the predicted sizes of cleavage

- 1 products after cleavage by target ribozymes are given
 2 below in Table 1.

3

TABLE 1

	Restriction Enzyme	RNA Size	Cleavage Products
Example 1 <i>rhodopsin</i> Mouse rhodopsin Mouse rhodopsin hybrid 1 Mouse rhodopsin hybrid 2 Rib 3 (See Table 1; sequences 1-4; Figures 1-6; Figures 1-6)	Eco47111 Eco47111 Fsp 1 Xho 1	778 bases 643 bases 577 bases 60 bases	336 + 442 bases
Example 2 Human rhodopsin Human rhodopsin hybrid Rib 15 (See Table 1; sequences 5-7; figures 7-11)	BstEII Acy 1 BstEII Acy 1 FspI XbaI	8511 bases 1183 bases 841 bases 1173 bases 300 bases 55 bases	61 + 790 bases 61 + 1122 bases
Example 3 Mouse peripherin Mouse peripherin hybrid Rib 17 (See Table 1; sequences 8-10; figures 12-15)	BglI BglI XbaI	488 bases 344 bases 60 bases	201 + 287 bases
Example 4 Human peripherin Human peripherin hybrid Rib 8 Rib 9 (see Table 1; sequence 11-14; figures 16-19)	BglI AvrII XbaI XbaI	489 bases 331 bases 55 bases 55 bases	238 + 251 (Rib 8) 194 + 295 (Rib 9)
Example 5 Collagen 1A2 Rib 18 (See Table 1; sequences 15 and 16)	XhoI XbaI		
Example 6 Antisense constructs (See Table 1; sequences 17 and 18)			

- 3 The examples of the invention are illustrated in the

1 accompanying figures wherein:

2
3 ~~Diagram~~ ^{Figure} 1 pBR322 was cut with MspI, radioactively
4 labeled and run on a polyacrylamide gel to enable
5 separation of the resulting DNA fragments. The sizes of
6 these fragments are given in ~~Diagram~~ ^{Figure} 1. This DNA ladder
7 was then used on subsequent polyacrylamide gels to
8 provide an estimate of the size of the RNA products run
9 on the gels.

10.

11 Figure ~~1~~ ²

12 A: Mouse rhodopsin cDNA was expressed from the T7
13 promoter to the Eco47III site in the coding sequence.
14 The RNA was mixed with Rib3/RNA with varying
15 concentrations of magnesium chloride. Lane 1-4:
16 Rhodopsin RNA and Rib3/RNA after incubation for 3 hours
17 at 37°C with 0mM, 5mM, 10mM and 15mM magnesium
18 chloride. The sizes of the expressed RNAs and cleavage
19 products are as expected (Table 1). Complete cleavage
20 of mouse rhodopsin RNA was obtained with a small
21 residual amount of intact RNA present at 5mM magnesium
22 chloride. Note at 0mM magnesium chloride before
23 activation of Rib3, ^{RNA} no cleavage products were observed.

24

25 B: Mouse rhodopsin cDNA was expressed from the T7
26 promoter to the Eco47III site in the coding sequence.
27 Resulting RNA was mixed with Rib3/RNA with varying
28 concentrations of magnesium chloride. Lane 1: DNA
29 ladder as in ~~Diagram~~ ^{Figure} 1. Lane 2: intact mouse rhodopsin
30 RNA. Lane 3-6: Rhodopsin RNA and Rib3/RNA after
31 incubation for 3 hours at 37°C with 0mM, 5mM, 10mM and
32 15mM magnesium chloride. Again complete cleavage of
33 mouse rhodopsin RNA was obtained with a small residual
34 amount of intact RNA present at 5mM magnesium chloride.
35 Lane 7: DNA ladder as in ~~Diagram~~ ^{Figure} 1.

36

RNA

✓ Figure

Figure 25

Mouse rhodopsin cDNA with altered 5'UTR sequence was expressed from the T7 promoter to the Eco47III site in the coding sequence. The resulting RNA was mixed with Rib3 RNA with 10mM magnesium chloride and incubated at 37°C. Lane 1: intact altered mouse rhodopsin RNAs. Lane 2-6: altered mouse rhodopsin RNA and Rib3 RNA after incubation for 0, 30, 60, 120, 180 minutes. No cleavage of the hybrid RNA was obtained. Notably after 3 hours incubation with Rib3 the adapted mouse rhodopsin RNA was as intense as at 0 minutes. Lane 7: DNA ladder as

1 *Figure*
in Diagram 1.

2

3 *Figure 6*

4 A: The unadapted mouse rhodopsin cDNA and the mouse
5 rhodopsin cDNA with altered 5'UTR sequence were
6 expressed from the T7 promoter to the Eco47III site in
7 the coding sequence. The resulting RNAs were mixed
8 together with Rib3 RNA and 10mM magnesium chloride. Lane
9 1: intact unadapted and altered mouse rhodopsin RNAs
10 which can clearly be differentiated by size as
11 predicted (Table 1). Lane 2-6: unadapted and altered
12 mouse rhodopsin RNAs and Rib3 RNA after incubation for
13 0, 30, 60, 120, 180 minutes with 10mM magnesium chloride
14 at 37°C. No cleavage of the altered hybrid RNA was
15 obtained. The hybrid was of equal intensity after 3
16 hours as it was at 0 minutes. Notably the majority of
17 the unadapted mouse rhodopsin RNA is cleaved
18 immediately by Rib3 RNA in the presence of the altered
19 mouse rhodopsin RNA. The cleavage products are
20 highlighted with arrows. The background is due to a
21 small amount of RNA degradation. B: In a separate
22 experiment the three RNAs (unadapted, altered mouse
23 rhodopsin RNAs and Rib3 RNA) were incubated at 15mM
24 magnesium chloride for 5 hours. The altered hybrid RNA
25 remains intact but the unadapted mouse rhodopsin RNA
26 has been cleaved completely.

27

28 *Figure 7*

29 A second altered mouse rhodopsin cDNA involving a
30 single base change at the ribozyme cleavage site was
31 generated. This adapted mouse rhodopsin cDNA was
32 expressed from the T7 promoter to the FspI site in the
33 coding sequence. Likewise the unadapted mouse rhodopsin
34 cDNA was expressed from the T7 promoter to the Eco47III
35 site in the coding sequence. These two RNAs were mixed
36 with Rib3 RNA and incubated at 37°C with 15mM magnesium

1 chloride. Lane 1: Intact mouse rhodopsin RNA. Lane 2:
 2 Intact altered mouse rhodopsin RNA (2nd alteration).
 3 Lane 3: DNA ladder as in ~~Diagram 1~~ ^{Figure 1}. Lanes 4-7:
 4 Unadapted and altered mouse rhodopsin RNAs and Rib3^{RNA}
 5 after incubation for 0, 60, 120 and 180 minutes ~~with~~ ^{with}
 6 15mM magnesium chloride at 37°C. Note the reduction of
 7 the unadapted RNA product over time in the presence of
 8 the altered RNA (Lanes 4 and 5). The adapted RNA
 9 remains intact and maintains equal ~~in~~ intensity at each
 10 time point indicating that it is resistant to cleavage
 11 by Rib3^{RNA}. Again, ^{as} with all other altered RNAs, no
 12 additional cleavage products were observed. Lane 8: The
 13 unadapted and adapted mouse rhodopsin ^{RNA} without ribozyme.
 14 Lane 9: DNA ladder as in ~~Diagram 1~~ ^{Figure 1}.

15
 16 Figure 2⁸
 17 Human rhodopsin was expressed from the T7 promoter to
 18 the BstEII site in Exon IV. The resulting RNA was mixed
 19 with Rib15^{RNA} with varying concentrations of magnesium
 20 chloride. Lane 1: intact rhodopsin RNA alone. Lane 2:
 21 Rib15 alone. Lane 3: DNA ladder as in ~~Diagram 1~~ ^{Figure 1}. Lanes
 22 4-7: Rhodopsin RNA and Rib15^{RNA} after incubation for 3
 23 hours at 37°C with the 0mM, 5mM, 10mM and 15mM
 24 magnesium chloride. Predicted cleavage products are 61
 25 and 790 bases (Table 1). Lane 8: DNA ladder. Partial
 26 cleavage of the RNA was obtained - a doublet
 27 representing the intact RNA and the larger cleavage
 28 product is present (most clearly in lane 5). The gel
 29 was run a shorter distance than the gel presented in
 30 Figure ~~8-11~~ ⁹⁻¹² to show the presence of Rib15^{RNA} at the
 31 bottom of the gel and to demonstrate that one of the
 32 cleavage products cannot be ~~visualized~~ ^{visualized} due to the presence
 33 of the labeled ribozyme which runs at approximately the
 34 same size. Subsequent gels were run further to achieve
 35 better separation of these two RNA fragments.

36

1 Figure 8⁹
2 Both the unadapted human rhodopsin cDNA and the altered
3 cDNA were expressed from the T7 promoter to the BstEII
4 site in Exon IV. The resulting RNA was mixed with
5 Rib15 RNA with varying concentrations of magnesium
6 chloride. Lane 1: intact human rhodopsin RNA alone.
7 Lane 2: DNA ladder as in ^{Figure}Diagram 1. Lane 3-6: Rhodopsin
8 RNA and Rib15 RNA after incubation together for 3 hours
9 at 37°C ^{with} 0mM, 5mM, 10mM and 15mM magnesium chloride.
10 Lane 7: DNA ladder as in ^{Figure}Diagram 1. Lane 8-11: Human
11 rhodopsin RNA with altered 5'UTR sequence and Rib15 RNA
12 after incubation together for 3 hours at 37°C with 0mM,
13 5mM, 10mM and 15mM magnesium chloride. Lane 12: intact
14 human rhodopsin RNA with altered 5'UTR sequence alone.
15 The predicted cleavage products for human rhodopsin are
16 61 and 790 bases (Table 1) - the larger cleavage
17 product is clearly visible when the ribozyme becomes
18 active after the addition of magnesium chloride (Lanes
19 4-6). This larger cleavage product is highlighted by an
20 arrow.

21
22 Figure 9¹⁰
23 Human rhodopsin cDNA was expressed from the T7 promoter
24 to the BstEII site in Exon IV. Likewise the altered
25 human rhodopsin cDNA was expressed from the T7 promoter
26 to the Fspl site in Exon 1. Both resulting RNAs were
27 mixed together ^{with} Rib15 RNA with varying concentrations
28 of magnesium chloride. Lane 1: DNA ladder as in ^{Figure}Diagram
29 1. Lanes 2-5: Rhodopsin RNA, altered rhodopsin RNA and
30 Rib15 RNA after incubation for 3 hours at 37°C with 0mM,
31 5mM, 10mM and 15mM magnesium chloride. The sizes of the
32 expressed RNAs and cleavage products are as expected
33 (Table 1). Partial cleavage of the unadapted RNA was
34 obtained after magnesium was added to the reaction. The
35 altered human rhodopsin RNA was protected from cleavage
36 in all reactions. If cleavage of the altered human

1 rhodopsin RNA had ~~occured~~^{occurred} the products rationally would
2 most likely be of a different size than those observed
3 with the unadapted RNA. Notably no additional cleavage
4 products were observed. Moreover there was no change in
5 intensity of the altered RNA when the ribozyme was
6 active (in the presence of magnesium chloride) or
7 inactive (at 0mM magnesium chloride). In contrast the
8 undapted human rhodopsin RNA is less intense in lanes
9 3-5 after cleavage than in lane 2 before the addition
10 of magnesium to activate Rib15. Lane 6: intact human
11 rhodopsin RNA. Lane 7: intact human rhodopsin RNA with
12 altered 5'UTR sequence. Lane 8: DNA ladder.

13

14 Figure 10/11

15 Human rhodopsin cDNA was expressed from the T7 promoter
16 to the BstEII site in Exon IV. Likewise the altered
17 human rhodopsin cDNA was expressed from the T7 promoter
18 to the Acyl in the 3'rhodopsin sequence after the stop
19 codon. Both resulting RNAs were mixed together with
20 Rib15RNA with varying concentrations of magnesium
21 chloride. Lane 1: DNA ladder as in ~~Diagram~~^{Figure} 1. Lanes
22 2-5: Rhodopsin RNA, altered rhodopsin RNA and Rib15RNA
23 after incubation for 3 hours at 37°C with 0mM, 5mM,
24 10mM and 15mM magnesium chloride. Lane 6: Intact human
25 rhodopsin RNA. Lane 7: DNA ladder as in ~~Diagram~~^{Figure} 1. Note
26 that neither RNAs or cleavage products are present in
27 Lane 5 as too little sample may have been loaded in
28 this lane.

29

30 Figure 11/12

31 Human rhodopsin cDNA and the cDNA with altered
32 5'sequence were expressed from the T7 promoter to the
33 Acyl site after the coding sequence of human rhodopsin.
34 The resulting RNA was mixed ~~with~~^{with} Rib15RNA with varying
35 concentrations of magnesium chloride. Lane 1: DNA
36 ladder as in ~~Diagram~~^{Figure} 1. Lane 2-5: Human rhodopsin RNA

1 and Rib15^{RNA} after incubation together for 3 hours at
2 37°C with 0mM, 5mM, 10mM and 15mM magnesium chloride.
3 Lane 6: Intact human rhodopsin RNA. Lane 7: DNA ladder
4 as in ^{Figure} ~~Diagram~~ 1. Lane 8-11: Human rhodopsin RNA with
5 altered 5'UTR sequence and Rib15^{RNA} after incubation
6 together for 3 hours at 37°C with 0mM, 5mM, 10mM and
7 15mM magnesium chloride. Lane 12: intact human
8 rhodopsin RNA with altered 5'UTR sequence alone. Lane
9 13: DNA ladder as in ^{Figure} ~~Diagram~~ 1. The larger of the
10 predicted cleavage products is present in lanes 3-5 and
11 is highlighted by an arrow. The adapted human rhodopsin
12 RNA again was protected from cleavage by Rib15^{RNA}. Note
13 that in Lane 12 too little sample may have been loaded.

14
15 Figure ~~22~~ 13
16 Mouse peripherin cDNA was expressed from the T7
17 promoter to the BgIII site in the coding sequence. The
18 RNA was mixed with Rib17^{RNA} with varying concentrations
19 of magnesium chloride. Lane 1: DNA ladder as in ^{Figure} ~~Diagram~~
20 1. Lane 2: intact mouse peripherin RNA. Lanes 3-6:
21 Mouse peripherin RNA and Rib17^{RNA} after incubation for
22 3 hours at 37°C ^{with} ~~0.0mM~~ 0.0mM, 5mM, 10mM and 15mM magnesium
23 chloride. The sizes of the expressed RNAs and cleavage
24 products are as expected (Table 1). Partial cleavage of
25 mouse rhodopsin RNA was obtained once Rib17^{RNA} was
26 activated with magnesium chloride. Possibly some of the
27 RNA was in a conformation that was inaccessible to
28 Rib17^{RNA}. It should be noted that in the ^{absence} ~~absence~~ of
29 magnesium chloride the ribozyme was inactive and no
30 cleavage products were observed.

31
32 Figure ~~13~~ 14
33 Mouse peripherin cDNA was expressed from the T7
34 promoter to the ^{Bgl II} ~~BgIII~~ site in the coding sequence. The
35 resulting RNA was mixed with Rib17^{RNA} with 15mM
36 magnesium chloride and incubated at 37°C for varying

1 times. Lane 1: DNA ladder as in ^{Figure}Diagram 1. Lane 2:
2 intact mouse peripherin RNA. Lanes 3-6: Mouse
3 peripherin RNA and Rib17 RNA after incubation together
4 with 15mM magnesium chloride at 37°C for 0, 1, 2 and 3
5 hours respectively. The sizes of the expressed RNAs and
6 cleavage products are as expected (Table 1). Partial
7 cleavage of mouse rhodopsin RNA was obtained with Rib17
8 after 1 hour. The proportion of the RNA cleaved
9 increased over time. The intensity of the mouse
10 rhodopsin RNA band decreased visibly on the gel by 3
11 hours and similarly the cleavage products visibly
12 increased in intensity. It is possible that further
13 cleavage might be obtained over longer time periods.
14 Lane 7: DNA ladder as in ^{Figure}Diagram 1.

15
16 Figure ~~14~~¹⁵
17 Mouse peripherin cDNA with altered 5' sequences was
18 expressed from the T7 promoter to the ^{Bgl II}~~Bgl II~~ site in the
19 coding sequence. The resulting RNA was mixed with
20 Rib17 RNA with varying concentrations of magnesium
21 chloride. Lane 1: intact altered mouse peripherin RNA
22 with no ribozyme. Lanes 2-5: Mouse peripherin RNA with
23 altered 5' sequence and Rib17 RNA after incubation for 3
24 hours at 37°C with 0mM, 5mM, 10mM and 15mM magnesium
25 chloride. The sizes of the expressed RNAs are as
26 expected (Table 1). No cleavage of the adapted mouse
27 rhodopsin RNA was obtained before or after Rib17 ^{RNA} was
28 activated with magnesium chloride. Lane 6: DNA ladder
29 as in ^{Figure}Diagram 1.

30
31 Figure ~~15~~¹⁶
32 Both the unadapted and adapted mouse peripherin cDNAs
33 were expressed from the T7 promoter to the ^{Bgl II}~~Bgl II~~ site
34 in the coding ^{sequence}~~sequence~~. The resulting RNAs were mixed
35 together with Rib17 RNA with 15mM magnesium chloride and
36 incubated at 37°C for varying times. Lane 1: DNA ladder

1 as in ^{Figure} ~~Diagram~~ 1. Lane 2: intact unadapted and altered
2 mouse peripherin RNA. Lanes 3-6: Unadapted mouse
3 peripherin RNA, altered mouse peripherin RNA and
4 Rib17^{RNA} after incubation together with 15mM magnesium
5 chloride at 37°C for 0, 30, 90 and 180 minutes
6 respectively. The sizes of the expressed RNAs and
7 cleavage products are as expected (Table 1). Partial
8 cleavage of the unadapted mouse peripherin RNA was
9 obtained with Rib17^{RNA} after 1 hour. The intensity of the
10 larger unadapted mouse peripherin RNA product decreases
11 slightly over time. In contrast the cleavage products
12 increase in intensity. The intensity of the smaller
13 altered mouse peripherin RNA product remains constant
14 over time indicating that the RNA is not cleaved by
15 Rib17^{RNA}. Lane 7: DNA ladder as in ^{Figure} ~~Diagram~~ 1.

16
17 Figure ~~26~~ 17

18 Both the unadapted and adapted human peripherin cDNAs
19 were expressed from the T7 promoter to the BglIII site
20 in the coding sequence. The resulting RNAs were mixed
21 together with Rib8 RNA with varying ^{concentrations} ~~concentrations~~ of
22 magnesium chloride and incubated at 37°C for 3 hours.
23 Lane 1: Unadapted human peripherin without ribozyme.
24 Lanes 2-5: Unadapted human peripherin RNA and Rib8 RNA
25 after incubation together with 0, 5, 10, 15mM magnesium
26 chloride respectively at 37°C for 3 hours. The sizes of
27 the expressed RNAs and cleavage products are as
28 expected (Table 1). Almost complete cleavage of the
29 unadapted human peripherin RNA was obtained with Rib8^{RNA}
30 after 3 hours. The intensity of the larger unadapted
31 human peripherin RNA product decreases over time. Lanes
32 6-9: Altered human peripherin RNA and Rib8 RNA after
33 incubation together ^{with} ~~with~~ 0, 5, 10, 15mM magnesium chloride
34 respectively at 37°C for 3 hours. The sizes of the
35 expressed RNAs are as expected (Table 1). No cleavage
36 of the alter d human peripherin RNA was obtained with

1 Rib8 ^{RNA} even after 3 hours. The intensity of the smaller
2 altered mouse peripherin RNA product remains constant
3 (with the exception of lane 9 in which less sample may
4 have been loaded) indicating that the RNA is not
5 cleaved by Rib8. ^{RNA} In addition no cleavage products were
6 observed. Lane 10: Intact unadapted human peripherin
7 RNA alone. Lane 11: Intact altered human peripherin RNA
8 alone. Lane 12: DNA ladder as in ^{Figure 1} ~~Diagram~~ 1.

9
10 Figure 17/18

11 The unadapted and altered human peripherin cDNAs were
12 expressed from the T7 promoter to the BglIII site in the
13 coding sequence. The resulting RNAs were mixed together
14 with Rib8 RNA for varying times with 15mM magnesium
15 chloride and incubated at 37°C. Lane 1: DNA ladder as
16 in ^{Figure} ~~Diagram~~ 1. Lane 2-5: Unadapted and altered human
17 peripherin RNAs and Rib8 RNA after incubation together
18 for 0, 1, 2 and 3 hours respectively at 37°C with 15mM
19 magnesium chloride. The sizes of the expressed RNAs and
20 cleavage products are as expected (Table 1). Almost
21 complete cleavage of the larger unadapted human
22 peripherin RNA was ^{obtained} ~~obtained~~ with Rib8 ^{RNA} after 3 hours.
23 The intensity of the larger unadapted human peripherin
24 RNA product decreases over time. Altered human
25 peripherin RNA was not cleaved by Rib8 ^{RNA} even after 3
26 hours. The intensity of the smaller altered mouse
27 peripherin RNA product remains constant over time
28 indicating that the RNA is not cleaved by Rib8 ^{RNA}. In
29 addition no additional cleavage products were observed.
30 Lane 6: Intact unadapted and altered human peripherin
31 RNA together without ribozyme. Lane 7: DNA ladder as in
32 ^{Figure} ~~Diagram~~ 1.

33
34 Figure 18/19

35 Both the unadapted and adapted human peripherin cDNAs
36 were expressed from the T7 promoter to the BglIII site

1 in the coding sequence. The resulting RNAs were mixed
 2 together with Rib9 RNA with varying ^{concentrations} concentrations of
 3 magnesium chloride and incubated at 37°C for 3 hours.
 4 Lane 1: DNA ladder as in ^{Figure} Diagram 1. Lanes 2-5:
 5 Unadapted human peripherin RNA and Rib9 RNA after
 6 incubation together with 0, 5, 10, 15mM magnesium
 7 chloride respectively at 37°C for 3 hours. The sizes of
 8 the expressed RNAs and cleavage products are as
 9 expected (Table 1). Almost complete cleavage of the
 10 unadapted human peripherin RNA was obtained with Rib9^{RNA}.
 11 The intensity of the larger unadapted human peripherin
 12 RNA product decreases greatly. Lanes 6-9: Altered human
 13 peripherin RNA and Rib9 RNA after incubation together
 14 with 0, 5, 10, 15mM magnesium chloride respectively at
 15 37°C for 3 hours. The sizes of the expressed RNAs are
 16 as expected (Table 1). No cleavage of the altered human
 17 peripherin RNA was obtained with Rib17^{RNA} even after 3
 18 hours. The intensity of the smaller altered mouse
 19 peripherin RNA was observed - the product remains
 20 constant over time indicating that the RNA is not
 21 cleaved by Rib9^{RNA}. Lane 10: Intact unadapted human
 22 peripherin RNA alone. Lane 11: Intact altered human
 23 peripherin RNA alone. Lane 12: DNA ladder as in ^{Figure} Diagram
 24 1. Rib9^{RNA} was ^{designed} designed to target a different loop
 25 structure in the 5' sequence of human peripherin. It may
 26 result in slightly more efficient cleavage of RNA than
 27 Rib8^{RNA}.

28
29 Figure 18²⁰

30 The unadapted and altered human peripherin cDNAs were
 31 expressed from the T7 promoter to the ^{Bgl II} Bgl II site in the
 32 coding sequence. The resulting RNAs were mixed together
 33 with Rib9 RNA for varying times with 15mM magnesium
 34 chloride and incubated at 37°C. Lane 1: Intact
 35 unadapted human peripherin RNA without ribozyme. ^{Lane} Lane
 36 2: Intact altered human peripherin RNA without

1 ribozyme. Lanes 3 and 4: DNA ladder as in ^{Figure} ~~Diagram~~ 1.
 2 Lane 5-8: Unadapted and altered human peripherin RNAs
 3 and Rib9 RNA after incubation together for 0, 1, 2 and 3
 4 hours ^{respectively} at 37°C with 15mM magnesium
 5 chloride. The sizes of the expressed RNAs and cleavage
 6 products are as expected (Table 1). Cleavage products
 7 were observed at time zero. Almost complete cleavage of
 8 the larger unadapted human peripherin RNA was obtained
 9 with Rib9^{RNA} after 1 hour. The intensity of the larger
 10 unadapted human peripherin RNA product decreased
 11 quickly over time. The altered human peripherin RNA was
 12 not cleaved by Rib9^{RNA} even after 3 hours. The intensity
 13 of the smaller altered human peripherin RNA product
 14 remains constant over time indicating that the RNA is
 15 not cleaved by Rib9^{RNA}. In addition ^{no} additional cleavage
 16 products were observed. Lane 9: Intact unadapted and
 17 altered human peripherin RNA ^{together} without ribozyme.
 18 Lane 10: DNA ladder as in ^{Figure} ~~Diagram~~ 1.

20 Example 1

22 Mouse Rhodopsin

23 Rib3^{RNA} targeting the mouse rhodopsin 5' non-coding
 24 sequence was cut with Xho I and expressed in vitro. The
 25 mouse rhodopsin cDNA and hybrid cDNA with altered
 26 5' non-
 27 coding sequence (with the human peripherin 5'UTR
 28 sequence in place of the mouse rhodopsin 5'UTR
 29 sequence) were cut with Eco47111, expressed and both
 30 RNAs mixed separately and together with Rib3 RNA to
 31 test for cleavage. RNAs were mixed with varying
 32 concentrations of MgCl₂ and for varying amounts of time
 33 to optimise cleavage of RNA by Rib3^{RNA} (Figures ²⁻⁷ ~~1-6~~).
 34 Likewise a second hybrid with a small modification of
 35 the 5'UTR ^{sequence} ~~sequence~~ was cut with FspI, expressed and
 36 tested for cleavage with Rib3 RNA alone and together

1 with the original unadapted mouse rhodopsin RNA. This
2 alteration is a single base change at the ribozyme
3 cleavage site involving a U-->G, that is, altering the
4 ribozyme cleavage site from GUC to GGC thereby removing
5 the target site. In all cases the expressed RNA was the
6 correct size. In all cases cleavage of the larger
7 unadapted mouse rhodopsin RNA product was achieved. In
8 some cases cleavage was complete and all cleavage
9 products were of the predicted size. Notably hybrid
10 mouse rhodopsin RNAs with altered 5'UTR sequences were
11 not cleaved by Rib3 RNA either when mixed alone with
12 Rib3 RNA or when combined with Rib3 RNA and the
13 unadapted mouse rhodopsin RNA (Figures ~~1-6~~²⁻⁷). This
14 highlights the sequence specificity of the Rib3^{RNA} target
15 in that small sequence alterations may be all that is
16 required to prevent cleavage. Likewise small
17 modifications in the targets for the antisense arms of
18 ribozymes or more generally for any antisense may
19 result in the inability of a suppression effector to
20 attack the modified RNA. The first hybrid described
21 above could be used to prevent ribozyme cleavage or
22 antisense binding of many ribozymes or antisense
23 suppression effectors and therefore would be
24 particularly useful if more than one suppression
25 effector was required to achieve suppression.

27 Example 2

29 Human Rhodopsin

30 The human rhodopsin cDNA clone (with a full length
31 5'UTR) and the human rhodopsin hybrid cDNA clone with
32 altered 5'non-coding sequence (shorter 5'UTR) were cut
33 with BstEII and expressed in vitro. The Rib15 clone was
34 cut with XbaI and expressed in vitro. The resulting
35 ribozyme and human rhodopsin RNAs were mixed with
36 varying concentrations of MgCl₂ to ^{optimize} ~~optimize~~ cleavage of

the template RNA by Rib15^{428 RNA} (Figures ⁸⁻¹²~~7-11~~). The human rhodopsin cDNA and hybrid cDNA with altered 5'non-coding sequence were cut with Acyl, expressed and both RNAs mixed separately (due to their similar sizes) with Rib15 RNA to test for cleavage (Figures ⁸⁻¹²~~7-11~~). The human rhodopsin cDNA was cut with BstEII and the hybrid cDNA with altered 5'non-coding sequence cut with FspI, expressed and mixed separately and ⁸⁻¹²together with Rib15 RNA to test for cleavage (Figures ~~7-11~~). In all cases the expressed RNA was the correct size. Similarly in all cases the unadapted RNA template was cut into cleavage products of the predicted sizes. The cleavage of the unadapted RNA template was incomplete with some residual uncleaved RNA remaining. This may be due, for example, to the inability of the ribozyme to access RNA in some conformations. In all cases RNA expressed from the altered hybrid human rhodopsin cDNA with a shorter 5'UTR remained intact, that is, it was not cleaved by Rib15^{RNA}. It is worth noting that Acyl enzyme cuts after the stop codon of the coding region of the gene and therefore the resulting RNA includes all of the coding sequence that gives rise to the protein. The RNA from the original unadapted human rhodopsin cDNA clone cut with Acyl is cleaved by Rib15^{RNA}. In contrast, RNA from the hybrid clone with an altered 5'UTR sequence is not cleaved by Rib15^{RNA} (Figures ⁸⁻¹²~~7-11~~). The sequence of the ribozyme target site and of the antisense flanks are not present in the altered human rhodopsin RNA. Clearly, altering the sequence in non-coding regions masks the resulting altered gene from being suppressed by antisense or ribozymes targeting sites in non-coding regions.

Example 3

Mouse Peripherin

1 Rib17 targeting mouse peripherin 5'^{italics}non-coding sequence
2 was cut with XbaI and expressed in vitro. The mouse
3 peripherin cDNA and mouse peripherin hybrid cDNA with
4 an altered 5'non-coding sequence (in which the mouse
5 peripherin 5'UTR sequence has been replaced by mouse
6 rhodopsin 5'UTR sequence) were cut with BglII, ^{BglII}
7 expressed in vitro^{italics} and both RNAs mixed separately and
8 together with Rib17 RNA to test for cleavage. RNAs were
9 mixed with varying concentrations of MgCl₂ and for
10 varying times to ^{optimize} cleavage of RNAs by Rib17^{RNA}
11 (Figures ¹³⁻¹⁶~~12-15~~). Partial cleavage of the unadapted
12 mouse peripherin RNA by Rib17^{RNA} was obtained - all RNAs
13 expressed and all cleavage products were the predicted
14 sizes. Partial cleavage may be due to the
15 inaccessibility of some RNA conformations to antisense
16 binding and/or ribozyme cleavage. In contrast the
17 adapted hybrid mouse peripherin RNA containing mouse
18 rhodopsin non-coding sequences remained intact (Figures
19 ¹³⁻¹⁶~~12-15~~). This again highlights that RNAs can be designed
20 so that they code for a correct protein, in this case,
21 mouse peripherin and such that they are masked from a
22 suppression effector(s), in this case, a ribozyme with
23 antisense flanks.

24 Example 4

25 Human Peripherin

26
27 Rib8 and Rib9 clones targeting human peripherin
28 5'non-coding sequence were cut with XbaI and expressed
29 in vitro. The human peripherin cDNA and human
30 peripherin hybrid cDNA with altered 5'non-coding
31 sequence (with part of the human peripherin 5'UTR
32 sequence replaced by mouse peripherin 5'UTR sequence)
33 were cut with BglII and AvrII respectively, expressed
34 in vitro and both RNAs mixed separately and together
35 with Rib9 RNA to test for cleavage. RNAs were mixed
36

1 with varying concentrations of MgCl₂ to optimise
 2 cleavage of RNAs by Rib9^{RNA} (Figures ¹⁷⁻²⁰16-19). Notably the
 3 majority of the larger unadapted RNA product was
 4 cleaved while the adapted RNA product with altered non-
 5 coding sequence remained intact (Figures ¹⁷⁻²⁰16-19).
 6 Similar results were obtained with Rib8^{RNA} which targets a
 7 different open loop than Rib9^{RNA} in the non-coding
 8 sequence of human peripherin. However, in the case of
 9 Rib8^{RNA} the extent of the cleavage was significantly less
 10 than Rib9^{RNA} (Figures ¹⁷⁻²⁰16-19) suggesting the important role
 11 of RNA structure in antisense binding and RNA cleavage.

12 Example 5

13 Human COL1A2

14
 15 Rib18 which has been cloned into [pCDNA3] ^{SEQ ID NO: 1} (Sequence 16)
 16 targets the 5'UTR sequence of the human type I collagen
 17 COL1A2 gene, multiple mutations in which can cause
 18 autosomal dominantly inherited osteogenesis imperfecta
 19 involving bone fragility amongst other symptoms. A
 20 clone containing the 5'UTR sequence together with exon
 21 I of the human COL1A2 gene has also been generated
 22 ^{SEQ ID NO: 1} (Sequence 15) to apply suppression and replacement
 23 strategies to this human gene.

24 Antisense constructs

25
 26 A number of constructs have been generated in [pCDNA3] ^{SEQ ID NO: 3} pCDNA3
 27 and pZEOSV containing tracks of sense and antisense
 28 sequence from the non-coding regions of the mouse
 29 rhodopsin and peripherin genes. An example of these
 30 sequences is given in ^{SEQ ID NOS: 17 and 18} (Sequences 17 and 18). Antisense
 31 effects are under evaluation.

32 DISCUSSION

33
 34 In the first four examples outlined above, RNA was

1 expressed from cDNAs coding for four different
2 proteins: mouse and human rhodopsin and mouse and human
3 peripherin. All four RNAs have been significantly
4 attacked in vitro using suppression effectors directed
5 towards the non-coding regions of the RNA. In all four
6 examples the ribozymes directed to 5'UTR sequences were
7 successful in cleaving target RNAs in the predicted
8 manner. Antisense targeting non-coding sequences was
9 used successfully to elicit binding and cleavage of
10 target RNAs in a sequence specific manner.

11
12 In some cases it is possible that cleavage of the RNA
13 at the 5'UTR would not effect the functioning of the
14 resulting RNA cleavage products in generating protein.
15 Moreover although lowering RNA levels may often lead to
16 a parallel lowering of protein levels this is not
17 always the case. In some situations mechanisms may
18 prevent a significant decrease in protein levels
19 despite a substantial decrease in levels of RNA.
20 However in many instances suppression at the RNA level
21 has been shown to be effective (see prior art). In some
22 cases it is thought that ribozymes elicit suppression
23 not only by cleavage of RNA but also by an antisense
24 effect due to the antisense arms in the ribozyme.
25 Notably we have demonstrated sequence specific attack
26 of target RNAs in non-coding regions, which is an
27 important stage in gene suppression.

28
29 In the four examples provided ribozymes were designed
30 to target 5'UTR sequences, however, they could be
31 readily designed to target any non-coding sequences.
32 Suppression could be achieved using antisense or
33 ribozymes targeting for example, the 3'UTR sequences or
34 any combination of non-coding sequences.

35
36 Additionally, in all four examples, cDNAs with altered

1 sequences in the non-coding regions targeted by
2 ribozymes were generated. RNAs expressed from altered
3 cDNAs were protected entirely from cleavage due the
4 absence of the ribozyme target by each of the ribozymes
5 tested. Alterations involved replacement of UTR
6 sequence with UTR sequence from another gene expressed
7 in the same tissue or UTR sequence from the same gene
8 but from a different mammalian species (e.g. mouse
9 peripherin, human peripherin, mouse rhodopsin). In one
10 case the target site was deleted (human rhodopsin). Of
11 particular interest is the second mouse rhodopsin
12 hybrid cDNA for Rib3 which contains a single base
13 change thereby preventing RNA cleavage. In some cases
14 the non-coding sequences of a gene may be essential to
15 the overall efficient expression and functioning of the
16 gene. Therefore it may be useful to alter replacement
17 genes in subtle ways to prevent ribozyme cleavage or
18 nucleic acid binding. Changing a few nucleotides in
19 many instances may be sufficient to prevent nucleolytic
20 attack.

21
22 As highlighted before in this text using this invention
23 the same method of suppression (targeting non-coding
24 sequences) and gene replacement (using a gene with
25 altered non-coding sequences) may be used as a
26 therapeutic approach for any mutation within a given
27 gene.

28
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1 CLAIMS

2

3 1. A strategy for suppressing expression of an
4 endogenous gene, wherein said strategy comprises
5 providing suppression effectors able to bind to the
6 non-coding regions of a gene to be suppressed, to
7 prevent the functional expression thereof.

8

9 2. A strategy as claimed in claim 1 wherein the
10 suppression effectors are antisense nucleic acids.

11

12 3. A strategy as claimed in claim 1 or claim 2 wherein
13 non-coding regions include the transcribed but non-
14 translated regions of a gene.

15

16 4. A strategy as claimed in claim 2 or 3 wherein the
17 antisense nucleic acids are DNA or RNA, directed to
18 5' and/or 3' untranslated regions and/or to introns
19 and/or to control regions or to any combination of
20 such untranslated regions.

21

22 5. A strategy as claimed in any of the preceding
23 claims wherein strategy further employs ribozymes.

24

25 6. A strategy as claimed in any of the preceding
26 claims wherein the strategy further employs
27 nucleotides which form triple helix DNA.

28

29 7. A strategy as claimed in any of the preceding
30 claims wherein the suppression effectors are
31 incorporated into a vector.

32

33 8. A strategy as claimed in claim 7 wherein the vector
34 is chose from DNA plasmid vectors, RNA or DNA virus
35 vectors.

36

- 1 9. A strategy as claimed in claim 7 or 8 wherein the
2 vector is combined with lipids, polymers or other
3 derivatives.
- 4
- 5 10. The use of a strategy as claimed in any preceding
6 claim in the preparation of a medicament for the
7 treatment of an autosomal dominant disease.
- 8
- 9 11. A strategy as claimed in any of claims 1 to 9
10 wherein the gene includes promoter regions.
- 11
- 12 12. A strategy for suppressing an endogenous gene and
13 introducing a replacement gene, said strategy
14 comprising the steps of:
 - 15 1. providing antisense nucleic acid able to bind to
16 at least one non-coding or untranslated region of a
17 gene to be suppressed and
18
 - 19 2. providing genomic DNA or cDNA encoding a
20 replacement gene sequence,
21
- 22 wherein the antisense nucleic acid is unable to
23 bind to equivalent non-coding or untranslated
24 regions in the genomic DNA or cDNA to prevent
25 expression of the replacement gene sequence.
26
- 27
- 28 13. A strategy as claimed in claim 12 wherein control
29 sequences of the replacement nucleic acid belong to
30 a different mammalian species, a different human
31 gene or are similar but altered from those in the
32 gene to be suppressed and thus permit translation
33 of the part of the replacement nucleic acid to be
34 initiated.
- 35
- 36 14. Replacement nucleic acids for use in a strategy as

1 claimed in any of claims 1 to 9 or claims 11 to 13,
2 with altered non-coding sequences such that
3 replacement nucleic acids cannot be recognised by
4 naturally occurring endogenous suppressors present
5 in one or more individuals, animals or plants.
6

7 15. Replacement nucleic acids as claimed in claim 14
8 comprising altered non-coding sequences to provide
9 the wild type or equivalent gene product being at
10 least partially protected from suppression by
11 naturally occurring endogenous suppression
12 effectors.
13

14 16. The use of a vector or vectors containing
15 suppression effectors in the form of nucleic acids,
16 said nucleic acids being directed towards
17 untranslated regions or control sequences of the
18 target gene and vector(s) containing genomic DNA or
19 cDNA encoding a replacement gene sequence to which
20 nucleic acids for suppression are unable to bind,
21 in the preparation of a combined medicament for the
22 treatment of an autosomal dominant disease.
23

24 17. A method of treatment for a disease caused by an
25 endogenous mutant gene, said method comprising
26 sequential or concomitant introduction of (a)
27 antisense nucleic acids to the non-coding regions
28 of a gene to be suppressed; to the 5' and/or 3'
29 untranslated regions of a gene or intronic regions
30 or to the non-control regions of a gene to be
31 suppressed, (b) replacement gene sequence with
32 control sequences which allow it to be expressed.
33

34 18. A method of treatment as claimed in claim 17
35 wherein the nucleic acid for gene suppression is
36 administered before or after or at the same time as

- 1 the replacement gene is administered.
2
3 19. A kit for use in the treatment of a disease caused
4 by an endogenous mutation in a gene, the kit
5 comprising nucleic acids for suppression able to
6 bind to the 5' and / or 3' untranslated regions or
7 intronic regions or control regions of the gene to
8 be suppressed and a replacement nucleic acid to
9 replace the mutant gene having a control sequence
10 to allow it to be expressed.
11
12 20. A method of treatment as claimed in claim 17 or 18
13 wherein nucleotides can be administered as naked
14 DNA or RNA, with or without ribozymes and/or with
15 dendrimers.
16